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GREEN SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL ACTIVITIES OF SILVER NANOPARTICLES PREPARED FROM *PTEROCARPUS MARSUPIUM*

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ABSTRACT
Background: Green synthesis of silver nanoparticles has gained much interest among the scientific community because of their colossal applications. In this concern, Indian flora has yet to divulge innumerable sources of cost effective, ecofriendly compounds in preparing AgNPs. Consequently, the present study involves an efficient and sustainable route of AgNPs preparation using *P.marsupium* bark extract which is well adorned for their medicinal properties. **Objective:** This study aims to investigate characterization and evaluation of silver nanoparticles prepared by green synthesis method using *P.marsupium* bark. **Methodology:** The green synthesis of silver nanoparticles from *P.marsupium* bark extract was accomplished. Synthesized AgNPs were characterized by UV-Vis spectrophotometer, SEM, XRD and FTIR analysis. The antibacterial efficacy was determined by disc diffusion method and antioxidant activity by DPPH method. *In vitro* cytotoxicity effect of biosynthesized AgNPs in SKOV-3 ovarian cancer cell line by MTT assay was also carried out. **Results:** The UV-VIS spectrum of silver nanoparticles revealed a characteristic Surface Plasmon Resonance (SPR) peak at 435nm. SEM study showed a spherical shaped AgNPs and size of AgNPs were about 240nm. XRD photograph indicated AgNPs were in crystalline face-centered cubic structure and the size range of 15-30 nm, further confirming the result of SEM. FTIR result also showed that the synthesized nanoparticles were capped with bimolecular compounds which are responsible for the reduction of silver ions. The AgNPs nanoparticles have shown a potential antibacterial, antioxidant and cytotoxicity activities when compared with their respective standards. **Conclusion:** The present study confirms that the biosynthesized AgNPs from *P.marsupium* bark extract were found to be ecofriendly, easy, cost effective and have promising pharmacological activities.

KEYWORDS: *P.marsupium*, silver nanoparticles, characterization, disc diffusion assay, DPPH, SKOV-3 cells.

INTRODUCTION
Nanotechnology is emerging as a rapidly growing field for its application in science and technology for the purpose of manufacturing new materials at nano scale.[1] Its vast applications such as medical sector for imaging, faster diagnosis, drug delivery, tissue regeneration, cancer therapeutics, bactericidal agent, antioxidants are due to their unique optical, electronic, magnetic and chemical properties which could be attributed to their small size and surface-to-volume ratio.[2] The biosynthesis of nanoparticles using green technology has been proposed as cost effective, environmental friendly alternative to chemical and physical method.[3] The different parts of plant such as stem, root, fruit, seed, callus, peel, leaves and flower are used to synthesis of metallic nanoparticles in various shapes and sizes by biological approaches. Silver is well-known since ancient times due to its medicinal value and preservative properties. Silver is also the only material whose plasmon resonance can be tuned to any wavelength in the visible spectrum. Therefore, silver nanoparticles (AgNPs) are playing a major role in the field of nanomedicine. They have a potential use in catalysis, textile manufacture and microelectronics. Major biological applications include biotherapeutics, biomolecular diagnostics, drug delivery, food production, agriculture, antibacterial agents and waste treatment.[4]

Pterocarpus marsupium Roxb. is commonly known as Indian Kino (English), Bijasal (Hindi) and Raktahonne (Kannada).[5] It is native to India, Nepal and Sri Lanka, where it exists in parts of the Western Ghats .The heart wood, leaves, flowers and bark of the plant have useful medicinal properties.[6] It is widely used as an antidiabetic, hepatoprotective, anti-inflammatory,

antiulcer, antimicrobial and antioxidant. These widespread uses of *P.marsupium* are because of the presence of phytochemicals like flavonoids, terpenoids, polyphenols, saponins, tannins and alkaloids. These chemical constituents act as powerful pharmacological agent, hence this plant is used as a green source for large scale production of AgNPs[7] Bioreduction of silver ions to yield metal nanoparticles using plants such as *Cajanus cajan*,[8] *Cuminum cyminum*,[9] *Pisonia grandis*.[10] *Allium cepa*,[11] *Parthenium hysterophorus*[12] *Ocimum basillicum*,[13] *Murraya koenigii*[14] *Glycyrrhiza glabra*,[15] *Coriandrum sativum*[16] *and Ocimum sanctum*[17] have been reported. However, there are no reports of biological activities of AgNPs synthesis using *P.marsupium*. Taken literature into consideration, in this paper, we focused on recent developments in synthesis, characterization, bio-applications mainly on the antibacterial, antioxidant and anticancer properties of AgNPs in a single platform.

MATERIALS AND METHODS

Collection of plant material

Pterocarpus marsupium bark was collected from Kalaburagi district, Karnataka, during the month of June every year.

Preparation of bark extract

Freshly collected bark was washed thoroughly, shade dried and then powdered to required particle size. 20gm of bark powder was boiled in 100mL double distilled water for 20 minutes at 80˚C. After boiling, the extract was filtered using Whatmann No.1 (25µm pore size) filter paper. The filtrate was collected and stored at 4˚C for further use.

AgNPs Synthesis

5mL of the above extract was mixed with 95mL of deionized water.[18] To this, 1mM silver nitrate ($AgNO_3$) was added drop wise with constant stirring at room temperature till the colour of the solution changes from colourless to reddish brown. The whole mixture was kept at room temperature for 24 hours. The bioreduction of silver ions was monitored by periodic sampling by the UV spectrophotometer. The obtained AgNPs were purified through repeated centrifugation at 10,000 rpm for 20 minutes and pellet was used for characterization.

Characterization of synthesized silver nanoparticles[19]

A. UV-Visible Spectroscopy analysis

The bioreduction of Ag^+ ions in solution was monitored by UV-Visible spectrophotometer (Shimadzu UV–VIS 2450) at room temperature in the range of 200-800nm.Double distilled water was used as reference. The reaction mixture was diluted with deionized water and used for analysis.

B. FTIR analysis

FTIR spectra (Shimadzu FT-IR Prestige 21) of synthesized silver nanoparticles from *P.marsupium* bark extract were recorded by FTIR Affinity in the range 4000-400cm^{-1} to identify the functional group in the extract responsible for bioreduction.

C. X-ray Diffraction Analysis

The crystalline structure of the biosynthesized silver nanoparticles was investigated through X-ray diffraction technique using X-ray powder diffractometer. The silver nanoparticles dispersion was placed on a glass slide and the solution (ethanol) was allowed to evaporate, to get a thin film of silver nanoparticles. This thin film was subjected to X-ray diffraction operating between 10º and 80º with the scanning rate of 2º per minute.

D. SEM analysis

SEM is a surface imaging method, capable of resolving different particle sizes, size distributions, nanomaterial shapes and surface morphology of the synthesized particles at nanoscales.[20] Purified AgNPs were sonicated for 15 minutes to make it uniform distribution and a drop of this solution was loaded on carbon-coated copper grids and solvent was allowed to evaporate under infrared light for 30 minutes. The synthesized nanoparticles were examined by scanning electron microscope (JOEL JSM) to know the shape and size of the particles.

Antibacterial activity

Disc Diffusion Method

The bacterial isolates *Enterococcus faecalis*, *Staphylococcus aureus* and *Shigella dysenteriae* was grown in nutrient broth for 18 hours and standardized to 0.5 McFarland standards (106 CFU/mL). The nutrient agar plates were prepared by pouring 20mL of molten media into sterile petriplates.0.1% inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. Wells were punched using a sterile 6mm cork borer. Different concentrations (25µg/mL, 50µg/mL) of synthesized silver nanoparticles were added into the wells, incubated at 37ºC for 24 hours. The effects were compared with standard chemotherapeutic agent Nalidixic acid (30mcg), Amikacin (30mcg) and Piperacillin (30mcg) for *E. faecalis*, *S. aureus* and *S. dysenteriae* respectively. Antibacterial activity was assayed by measuring the diameter of inhibition zone formed around the well using standard (Hi-Media) scale. The experiment done in triplicates and the average values were calculated for antibacterial activity.[21]

Antioxidant activity

DPPH radical scavenging activity

The free radical scavenging activity of biosynthesized AgNPs against stable DPPH was performed using the method.[22] Briefly, 0.004%w/v of DPPH radical solution was prepared in methanol; 900µL of this solution was mixed with varying concentrations of AgNPs and standard ascorbic acid solution kept in dark place for 30

minutes. The absorbance was measured at 517nm. Scavenging capacity of DPPH radicals (%Inhibition) was measured by the following formula and finally calculated 50% inhibition concentration (IC_{50}) from the graph of inhibition percentage plotted against AgNPs concentration,

$$\text{DPPH radical scavenging activity (\%)} = (Ac - As)/Ac \times 100,$$

Where, Ac is the absorbance of control and As is the absorbance of test samples.

***In vitro* cytotoxicity activity**

MTT assay

Cell lines and culture medium:

Cell line was procured from ATCC, stock cells was cultured in medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100IU/mL), streptomycin (100µg/mL) in an humidified atmosphere of 5% CO_2 at 37ºC until confluent. The cells were dissociated with TPVG solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells are checked and centrifuged. Further, 50,000 cells / well of Jurkat was seeded in a 96 well plate and incubated for 24 hrs at 37ºC, 5 % CO_2 incubator.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µL of the diluted cell suspension (50,000cells/well) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µL of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 24hrs in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 100µL of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 hrs at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100µL of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

Calculating Inhibition: % Inhibition = 100 – (OD of sample/OD of Control) x 100.

IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, SanDiego, CA, USA).

Statistical analysis

Each experiment were repeated three times and the resulting bacterial growths on three plates corresponding to a particular sample were reported as the mean ± standard deviation (n = 3).

RESULTS AND DISCUSSION

Characterization of synthesized silver nanoparticles

A. UV-Visible Spectroscopy analysis

The addition of *P.marsupium* bark extract to silver nitrate ($AgNO_3$) solution resulted in colour change of the solution from yellow to reddish brown as shown in **Figure-1**, which is a preliminary confirmation of AgNPs formation. The colour change arises from the excitation of surface plasmon resonance (SPR). This property is largely governed and dependent upon the particle type, size, shape and the local chemical ambience. The characteristic fingerprint zone which exhibits this phenomenon (by the AgNPs) predominantly appears in the range of 400–500 nm respectively [23, 24]. Further confirmation of AgNPs synthesis was examined by UV–Vis spectrophotometer that produced a peak near 435nm which is specific for Ag nanoparticles as shown in **Figure-2**. Light wavelengths in the range of 300–800 nm are normally used for characterizing different metallic nanoparticles. The $AgNO_3$ solution and *P.marsupium* bark extract alone did not show any peak between 300–700 nm. Past studies in our laboratory suggested that a SPR peak located between 410-450nm has been observed for AgNPs.[25]

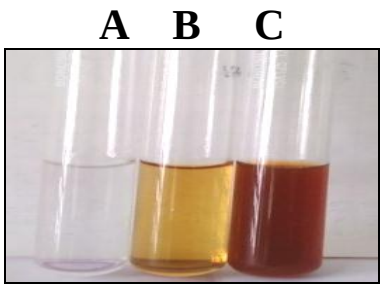

Figure-1: Sample tubes containing Silver nitrate solution (A), *P.marsupium* bark extract (B) and AgNPs solution (C).

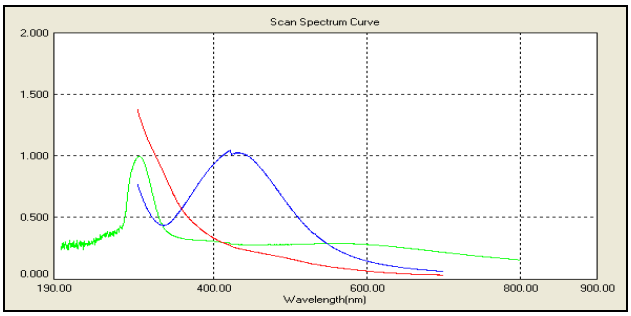

Figure-2: UV–Vis absorption peak of *P.marsupium* bark extract (green), $AgNO_3$ (red) and biosynthesized AgNPs (blue). The absorption spectrum of AgNPs displayed a strong (narrow) peak at 435nm which it is assigned to surface plasmon resonance of the particles.

B. FTIR analysis

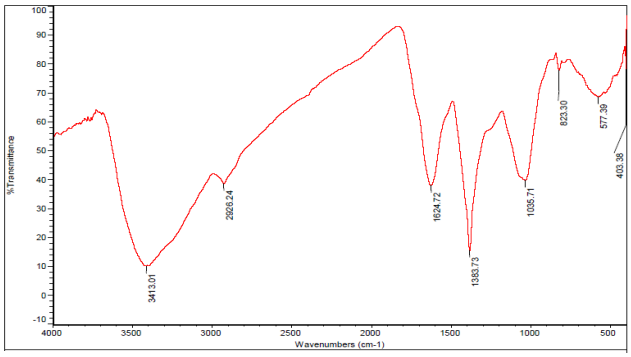

Figure-3: FTIR spectrum of biosynthesized silver nanoparticle from *P.marsupium*.

FTIR analysis were carried out to identify the biomolecules responsible for the reduction of silver ions and capping of the bioreduced silver nanoparticles synthesized by using plant extract. In **Figure-3**, FTIR spectra shows intense peak at 3413.01cm^{-1} corresponding to the N–H stretching frequency, which signifies the presence of protein. Another peak at 2962.24 signifies a single aldehyde group. The absorption peaks were also located at 1035.71, 1383.73 and 1624.72 in the region 500–4000cm^{-1}, corresponding to presence of fatty acids, carbonyl groups, flavanones and amide I band of proteins. These functional groups are having a role in maintaining stability of AgNPs as reported in earlier studies.[26]

C. X-ray Diffraction Analysis

The spectra were recorded in a Phillips Xpert Pro Diffractometer running at 40 kV and 30 mA. The diffracted intensities were also recorded and the calculation was performed with the help of instanano.com site which showed the presence of nanoparticles size of 1st peak was 30.44nm, 2nd peak was 30.75nm, 3rd peak was 31.26nm, 4th peak was 27.95nm, 5th peak was 30.37nm, 6th peak was 30.06nm and 7th peak was 15.08nm as depicted in **Figure-4**. Our result is in conformity with the reports of the Joint Committee on Powder Diffraction Standards (JCPDS No. 89-3722). The peaks were very sharp and clearly indicated that the AgNPs synthesized under nano-regime have crystalline nature with face-centered cubic phase. Similar observations were also recorded by earlier researchers.[27]

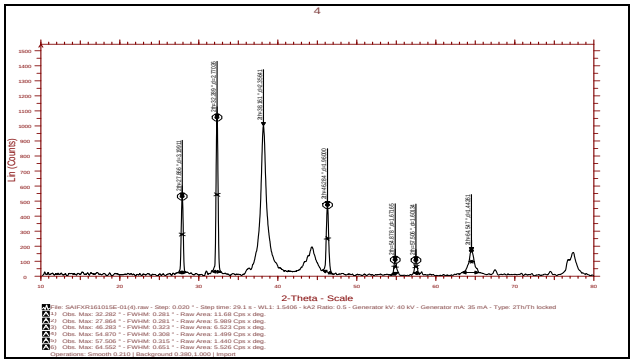

Figure-4: XRD diffractogram of biosynthesized silver nanoparticles.

D. SEM analysis

The average size of the synthesized silver nanoparticles from *P.marsupium* was about 240nm with spherical in shape as represented in **Figure-5**. Formation of silver nanoparticles was due to interactions of hydrogen bond and electrostatic interaction between the biomolecules capping with Ag. This is also supported by the shifts and difference in areas of the peaks obtained in the FTIR analysis.

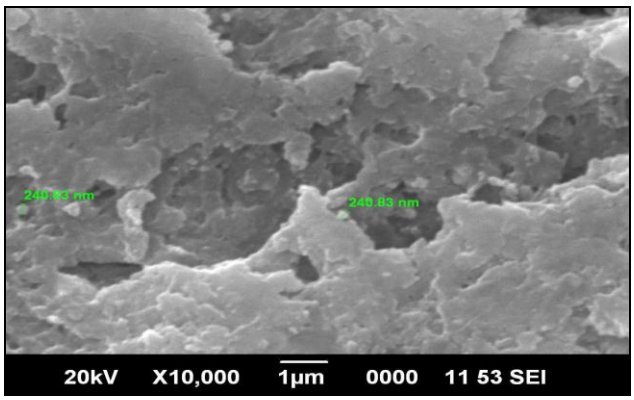

Figure-5: SEM image of biosynthesized AgNPs from *P.marsupium* bark extract.

Antibacterial activity

Disc Diffusion Method

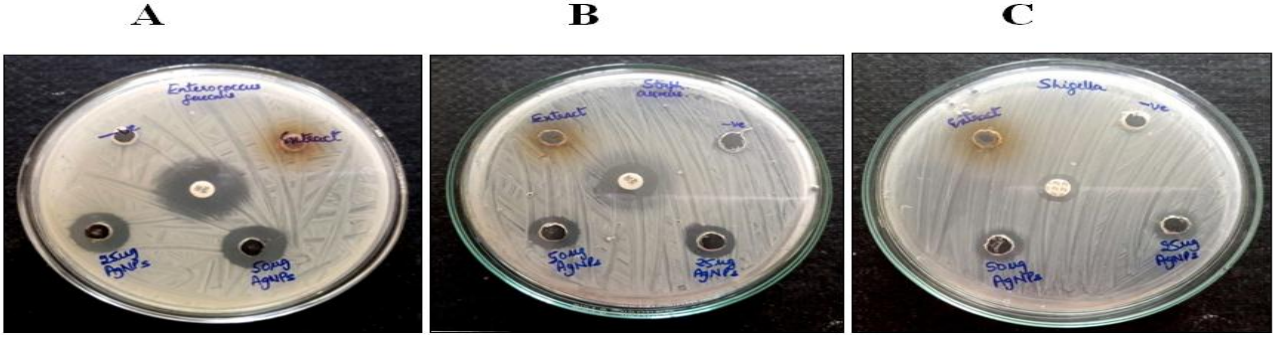

Figure-6: Antibacterial activity of AgNPs and *Pterocarpus marsupium* bark extract against A. *Enterococcus faecalis*, B. *Staphylococcus aureus*, C. *Shigella dysenteriae* respectively.

In recent years, nanoparticles have been considered as an interesting alternative to antibiotics and appear to have a high potential in solving bacterial multi-drug resistance in human pathogenic bacteria.[28] In present study, the synthesized AgNPs have displayed antibacterial activity against the pathogenic microbes as shown in **Figure-6**. The maximum zone of inhibition was observed with *E. faecalis* (17mm), followed by *S. aureus* (13mm) was observed at 50µg/mL concentration of AgNPs. The lowest zone of inhibition was found to be with *S. dysenteriae* (8mm) at 25µg/mL concentration of AgNPs. The synthesized AgNPs were found to have high inhibition activity when compared to the plant bark extract. AgNPs were more effective against Gram-positive bacteria than the Gram-negative bacteria which might be attributed by the membrane permeability. The potential reason for the antibacterial activity of AgNPs is that they attach to the surface of the cell membrane and penetrates inside the bacteria, disturbing the permeability and respiratory functions of the cell.[29]

Antioxidant activity

DPPH radical scavenging activity

DPPH assay mainly depends on the hydrogen donating capacity to scavenge DPPH radicals [30]. DPPH shows a characteristic absorption at 517nm whose colour changes from violet to yellow upon reduction and showed potent inhibitory capacity for the synthesized silver nanoparticles. The % of inhibition increased with increase in concentration of silver nanoparticles comparable with standard ascorbic acid and *P.marsupium* bark extract as shown in **Figure-7**. The antioxidant activity of silver nanoparticles is based on electron transfer reaction between Ag and 1,1-diphenyl-2-picryl hydrazyl radical. Silver nanoparticles quenched the activity of DPPH by donating electrons. These findings were comparable to the previously reported aqueous extracts of *Pterocarpus marsupium* that have showed a high inhibition at low concentration compared with standard reference.[31] Further, it is confirmed that silver nanoparticles posses a high potential antioxidant activity as compared to ascorbic acid with IC_{50} 3±0.23 for silver nanoparticles, 4±0.41 for ascorbic acid and 5±0.78 *P.marsupium* bark extract.

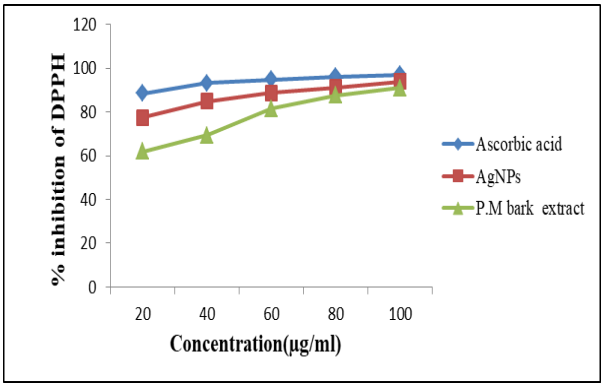

Figure-7: DPPH radical scavenging activities of ascorbic acid, AgNPs and *P.marsupium* bark extract. Values are Means ± SD (n = 3).

In vitro cytotoxicity activity

MTT assay

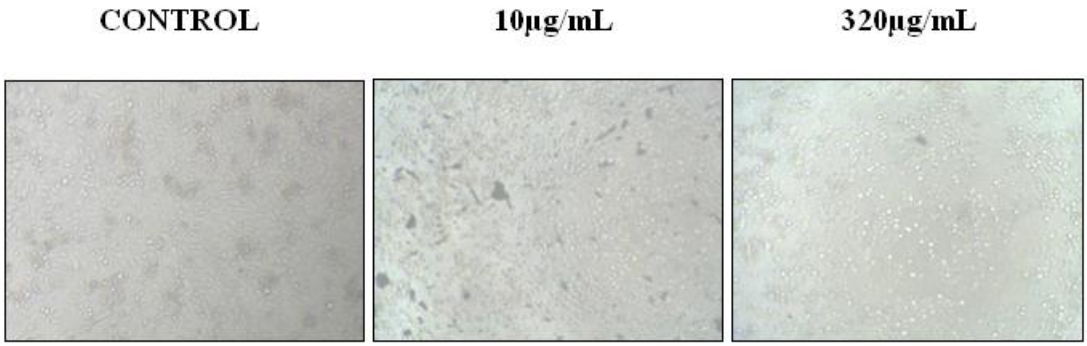

Figure-8: MTT Assay of AgNPs synthesized from *P.marsupium* bark extract.

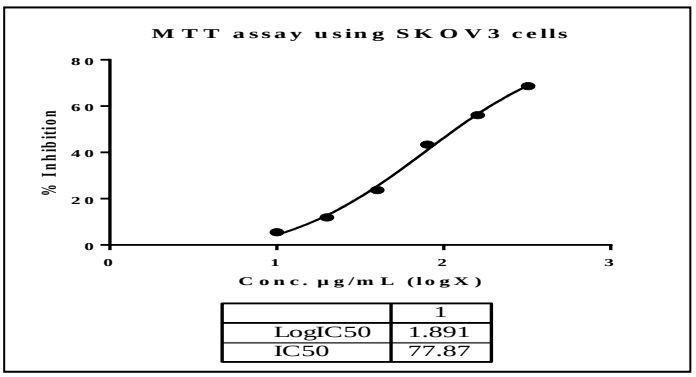

Figure-9: Cytotoxicity effect of AgNPs synthesized from *P.marsupium* bark extract.

Table-1: Percentage inhibition of AgNPs synthesized from *P.marsupium* bark extract.

SKOV3				
Compound name	**Conc. µg/mL**	**OD at 590nm**	**% Inhibition**	**IC_{50} µg/mL**
Control	0	0.641	0.00	
AgNPs	10	0.606	5.47	
	20	0.565	11.87	
	40	0.489	23.72	**77.87**
	80	0.363	43.38	
	160	0.282	56.08	
	320	0.201	68.65	

The SKOV-3 human ovarian carcinoma cells were exposed to AgNPs of *P.marsupium* at the concentrations of 0–360 µg/µL for 24hrs and the cytotoxicity was determined using MTT assays as depicted in **Figure-8**. The study of MTT assay results have shown that the above stated concentrations of AgNPs could significantly induce cytotoxicity in the SKOV-3 cells in a dose-dependent manner as represented in **Figure-9**. Data analysis of the cytotoxicity assay have shown that the IC_{50} values of AgNPs against the SKOV-3 cells were 77.87µg/µL after the incubation period as shown in **Table-1**($P<0.05$). These findings were in accordance with earlier report observed by several researchers.[32]

CONCLUSION

In this study, we have investigated eco-friendly and cost effective green silver nanoparticles synthesized from the bark extract of *P.marsupium*. The biosynthesized AgNPs demonstrated an excellent antibacterial, antioxidant and cytotoxicity activities. Hence, the AgNPs can be explored as a new source of alternative medicine for treating many human ailments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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